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(54) Title: THE BIOLOGICAL SELECTION OF USEFUL MOLECULES

(57) Abstract

A method for selecting a binding partner for a selector protein from among a library of proteins, comprises screening the library with a microorganism transformed such that it expresses the selector protein on its surface. By this method, an antibody recognising a protein can be isolated without more than partial knowledge of the encoding gene.

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THE BIOLOGICAL SELECTION OF USEFUL MOLECULES

Field of the Invention

The present invention relates to a biological method
to select useful molecules from libraries of chemical
specificities of a protein nature encoded by nucleic acid
sequences. In one embodiment, this method relies upon a
modification of the normal infectious interaction between
bacteriophage and bacterium. The invention utilises the
fact that there is a phage receptor for bacteria
(hereinafter "PRB") and a bacterial receptor for phage
(hereinafter "BRP").

Background of the Invention

It is known that peptides, proteins and antibody
domains may be expressed in bacteria or filamentous phage.
Vectors for this purpose include plasmids, phagemids and fd
or M13 derivatives.

It is also known to clone antibody variable regions by
PCR (polymerase chain reaction) technology from biological
tissues or cell lines, or by in vitro methods.

Protein epitopes can be expressed on the outer surface
of bacteria by cloning them, in frame, into different sites
within outer membrane proteins (OMPs), some of which act as
receptors for bacteriophages. That such cloned epitopes,
expressed in these systems, can be recognised by antibodies
is demonstrated by the fact that they have been used as
vaccines; see Charbit et al, Gene (1988) 70:181-189.

The infection of bacteria by bacteriophage involves
the interaction of a protein or other molecule (the PRB),
usually at one end of the bacteriophage (e.g. J protein in
lambda, gene III product in M13 and fd, gene 37 protein in
T4 phages, gene 38 protein in T2 and Ox2 phages), with a
protein or other molecule (the BRP) found on the surface of
the bacteria (e.g. lamB on E. coli for lambda, and a
component of the sex pilus on E. coli for M13 and fd, ompC
or lipopolysaccharide for T4 phages, ompA for Ox2, and ompF
for T2).

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The PRBs of different phages usually have a number of domains with different functions, only one of which is responsible for binding to the BRP. In the case of the J protein, for example, it has been shown that the N terminal part of the protein is responsible for assembly of the phage tail, while the C terminal domain is responsible for the host range. Similarly in the gene 37 protein of T4 phages, the BRP-recognising region of the PRB is found at the terminal end. In the case of fd, it is the N terminal part of the gene III protein which is responsible for binding to the F' pilus, while the C terminal has a structural role in virus assembly.

Boulain et al, Mol. Gen. Genet. (1986) 205:339-348, show that, in LamB, the BRP for phage lambda, the expression of epitopes in some sites disrupts the normal phage/bacterial interaction and inhibits infection, whereas other sites allow the expression of epitopes without affecting this interaction. Further, when the normal phage/bacterial interaction is inhibited by the expression of an epitope within the BRP (e.g. in lamB), this inhibition can be overcome by mutations within the PRB (the J protein). As the E. coli strains which these J gene host range mutants can infect is not limited to a unique peptide insertion within lamB, but apply to a number of different ones, it is likely that the normal PRB/BRP interaction has been substituted by an alternative one which is nevertheless found on the same proteins.

In some cases, evolutionary relationships can be seen between similar phages with different host ranges. In these cases, the PRB may retain some homology in the part required for phage assembly, but is usually different in the part responsible for the host range, e.g. lambda and 434, fd and IKe; see, respectively, Fuerst et al, Virol. (1978) 87: 437-458, and Peeters et al, J. Mol. Biol. (1985) 181: 27-39.

In some cases it has been possible to change host range specificity of bacteriophages in a predictable

fashion by exchanging the BRP binding domains between related bacteriophages. For example, exchanging the 3' region of the J gene in lambda with that of 434 (a related phage), in the absence of any other changes, gives the

5 recombinant lambda the host range of the 434 phage; see Fuerst et al (1978) supra.

Antibodies against the BRP can inhibit phage infection by interfering with phage binding. This has been shown, for lambda and K10 for monoclonal antibodies against lamB,

10 by Gabay et al, J. Biol. Chem. (1982) 257: 6627-6630.

Methods are available, to produce extremely diverse libraries of peptides; see, for example, Cwirle et al, PNAS USA (1990) 87: 6378-6382. The value of these libraries is that a large and important part of the epitope universe

15 (this may even be a complete repertoire of specificities) can be encompassed in a few microlitres of solution. These libraries are presently created by amplification of random sequences created by oligonucleotide synthesis, e.g. using PCR.

20 Marks et al, J. Mol Biol. (1991) 222: 581-597, describe the production of libraries of antibody domains in filamentous phage. The use of living microorganisms to produce the library is useful in that it allows easy amplification, manipulation and storage of the library and

25 the selected antibody once isolated.

The selection of appropriate phage antibodies from a library of such phage antibodies is presently performed by successive rounds of purification on a solid phase support. Phage peptides and antibodies of useful affinity can be

30 selected from a "naive" library (i.e. one from an unimmunised animal) using this method. An important aspect of this finding is that the DNA sequences encoding the phage antibody are physically linked to the antibody; consequently the DNA encoding the phage antibody is co-

35 purified with its binding specificity.

More generally, in the exploitation of such libraries (antibody or peptide), the selection procedure relies on

the use of a known molecule (hereinafter "the selector").
The selector is usually a solid-phase selector, i.e.
coupled to a solid-phase substrate (e.g. coated plate,
magnetic bead, tube or column). It is used to challenge
5   the library to select an element which binds to it
(hereinafter "the selected element").

In order to have knowledge of the selector, it must be
independently analysed and synthesised or purified, in
workable amounts. Both synthesis and purification are
10   generally expensive and inefficient. This is now realised
to be a problem that can be overcome.

To date, to derive an antibody, be it in bacterial or
myeloma cells, it is necessary to purify antigen in
workable amounts, a procedure which is not only inefficient
15   and expensive, but also often unique to each antigen. It
is clear that a procedure which is able to derive
antibodies against protein antigens utilising only the
cloned gene sequences encoding such an antigen would be a
breakthrough.

20   Summary of the Invention

The present invention is based on the realisation that
the specific molecular natures of the PRB and BRP are less
important for infection than the fact that they are able to
bind to one another, and that the living nature of a
25   library may also be exploited in the selection procedure,
by replacing the interaction of BRP with PRB with that of
an interacting antibody/antigen or other peptide
(protein)/peptide (protein) pair. The selector does not
need to be synthesised or purified; all that is required is
30   a DNA sequence which codes for the selector. DNA
sequences, either from cloned genes, libraries or randomly
generated, are far more easily manipulated and generated
than their corresponding peptide or protein counterparts.

More particularly, the present invention utilises the
35   normal productive interaction between bacteria and phage.
For example, desired antibody specificities expressed on
phage particles (phage antibodies) can be selected and

amplified from phage antibody libraries by biological means, without recourse to the experimental manipulation of any protein or peptide intermediates. By modification of the interaction between a bacterial cell and a phage, the normal BRP/PRB interaction, which leads to transfer of genetic information from phage to bacteria, is replaced with an antigen/antibody interaction (or indeed any other pair of proteins or peptides which are able to bind to one another), also leading to transfer of genetic information from phage to bacteria.

The permissivity of the PRB (gene III) in fd to foreign protein insertion is well documented and is being used to select useful molecules using the solid phase selector systems described above, whereas in the lambda system, the permissivity of the BRP (lamB) to foreign protein insertion is well documented and has been used to develop useful antibodies by classical methods. It is clear, therefore, that there are examples of successful manipulation of both BRP and PRB but in different bacterial phage systems. The fact that such manipulations can be performed in diverse heterologous systems indicates that manipulation of both partners of an infecting pair is possible.

Examples of suitable bacteria/phage pairs include: Ike, M13, f1 and fd with E. coli; T even phages with E. coli; RNA phages with E. coli; T odd phages with E. coli; lambdoid phages with E. coli with lambda. A particular interaction is based upon the expression of the antibody within the expression of the antigen within the lamB protein of E. coli.

The interaction may also be, for example, as for any cell virus pair. Examples of such cell/virus interactions include retroviruses with eukaryotic cells and Herpes viruses with eukaryotic cells.

Monoclonal antibodies have been derived which bind to the same part of the BRP as the PRB, suggesting that these could function as PRBs if placed within the correct context.

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The interaction may result in lysis of bacteria, in acquisition of a resistance phenotype by bacteria, or in the expression of any new gene in bacteria. It may occur in liquid culture or within or upon solid media.

Further novel procedures described herein reduce the size of antibody variable region domains, to alter the orientation of such domains when forming part of chimeric proteins. Others allow expression of functional antibody domains within the COOH terminal of chimeric proteins.

As an example of such a selective interaction, the proocedure may apply to the selection of an antibody or peptide (from a library of such molecules) interacting with the protein product of a cloned gene, without the need for synthesis or purification of the protein product of that gene. Antibodies against the protein products of genes may thus be derived directly from those genes or proteins. It is also clear that this serves as no more than one application of this procedure, and that the selection of any peptide or protein on the basis of its binding properties can be achieved.

Furthermore, the scheme proposed is not restricted to the selection of an element from a library by a single selector. As described below (scheme 3), the selector may be a library (restricted or general) and, by the confrontation of two such libraries, one expressed on phage, the other on bacteria, it is possible to select for pairs of interacting molecules, as well as for molecules which fail to interact with a given repertoire (scheme 3b). Particularly in this context, the invention allows selection (or discovery) of binding rather than of a binding partner as such.

While the PRB has been used to create libraries of diverse molecules (and to select binding partners from them) in the examples cited above, the present invention utilises the fact that the foreign proteins are expressed within the PRB (the use of solid phase selectors does not specifically require expression within the PRB).

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The use of living microorganisms to express libraries is useful in that it allows easy amplification, manipulation and storage of the library and the selected peptide or antibody once isolated. Furthermore, the DNA
5   sequences encoding the phage antibody are physically linked to the antibody or peptide, so that the DNA encoding the phage antibody or peptide is co-purified with its binding specificity.

The term antibody as used here refers to ScFv, dAb,
10   Fv, Fab, whole Ab and any derivatives thereof. Phage antibodies refer to any phage particle which expresses an antibody fused to the protein in the phage which acts as the phage receptor for bacteria (PRB).

Description of the Drawings
15   In the accompanying drawings:

Figures 1 to 3 each illustrate in a schematic way the starting materials, interactions and products of different procedures that illustrate independent embodiments of the invention, each embodiment being described below in more
20   detail under the corresponding Scheme heading; and

Figure 4 illustrates six alternative J gene/antibody fusion proteins that may be used in the invention.

More specifically, Fig. 1A shows bacteria as selector, Fig. 1B phage expressing a library of selectable elements
25   within the PRB, Fig. 1C the binding/non-binding of the phage to the bacteria, and Fig. 1D the result of growing infecting phage under lytic conditions. By contrast, Figs. 2A and 2B show bacteria expressing a library of selectable elements within the BRP and phage as selector, Fig. 2C the
30   binding/non-binding, and Fig. 2D the result of growing (ampicillin-resistant) lysogenic bacteria.

Figs. 3aA and 3bA are each the same as Fig. 2A, Figs. 3aB and 3bB the same as Fig. 1B. Figs 3aC and 3bC each show the confrontation of phage and bacterial libraries.
35   Fig. 3aD shows selected phage/bacterial pairs, Fig. 3aE the selected lysogenic bacterial colony from a single interaction, and Figs. 3aF and 3aG the respective results

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of amplification of lysogenic bacteria and infectious phage from each individual interaction. Fig. 3bD shows selected phage singles (confrontation under lysogenic or non-infectious conditions, leaving only non-binding or infectious particles). Fig. 3bE shows selected bacterial singles (confrontation under lytic conditions leads to survival and growth of non-infected bacteria).

Description of the Invention

The four schemes illustrated in the drawings are described, for the purposes of illustrating the invention only, as follows:

In the first scheme (see figure 1), the BRP is replaced by the selector and the PRB with a library of selectable elements encoded by DNA sequences inserted appropriately within the PRB. If the normal phage/bacterial infection mechanism is disrupted in this selection system, then the only way for infection, and hence phage amplification, to occur is by use of the selector/selected element interaction. If lytic phage is used in this scheme, only the selected element, in the form of phage, can be rescued following productive infection. This may be either as plaques after infection in a solid phase, or in supernatants after infection in a liquid phase. The use of non-lytic phage, similarly, will lead to isolation of the phage, either in the supernatant of a liquid phase after infection, or as turbid plaques or resistant colonies (depending upon the nature of the phage used), when plated after infection. In the latter case, the bacteria infected may also be isolated.

In the second scheme (see Fig. 2, using the transfer of antibiotic resistance as an example), the selector is found on the phage surface (within the PRB) and the library of selected elements on the bacterial cell surface (in the BRP). Selection will occur in a fashion similar to that described above, with the difference that one is selecting for a bacterial instead of a phage clone. It is clear that this mechanism will only work where phage infection confers

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a selectable phenotype (e.g. resistance to ampicillin, tetracycline, chloramphenicol or kanamycin) upon a bacterium, and that bacterium is able to grow as a bacterial clone. Lytic plaques will not occur as there will be no substrate for renewed infection by the phage (since adjacent bacteria will be different and not infectable). In this scheme, both selector and selected element can be recovered after productive infection: selected element by the growth of bacteria from the bacterial clone; selector by the use of appropriate temperature-sensitive phage mutants, or by the natural release of phage into the growth medium during growth of bacteria.

In what is essentially a combination of schemes 1 and 2, two libraries of specificities, one on phage and one on bacteria (within the PRB and BRP, respectively) may be confronted. The successful encounter of phage and bacteria will lead to plaques or colonies which will represent the productive binding of one element of each library (the selection of couples, Fg. 3a). Alternatively, elements not interacting with the library may be selected (the selection of singles, Fig. 3b).

The selector can be soluble, and act to protect the selected element (as expressed as part of the BRP) from interaction with a wild-type lytic phage. In this case, the selector is a soluble protein, the library of selected elements is expressed within the BRP on the surface of bacteria, and bacterial clones surviving after infection with wild-type phage will be expected to bind the selector.

All schemes are applicable to any pair of binding molecules, natural or engineered, and to the selection of elements of any interacting protein pair, of which antibody/antigen pairs are one of many. The demonstration that these schemes are able to work requires the substitution of a normal BRP/PRB pair with another pair of interacting molecules. The ways in which both sides of

diverse bacterial/phage system can be manipulated as required by this invention are described below for a number of different systems. The implementation of these schemes will be illustrated by reference to the selection of either

5 antibodies or antigens (and hence the DNA sequences encoding them), although it should be borne in mind that this is a technique which is applicable to the isolation of the DNA encoding one or both proteins involved in any binding interaction.

10 Scheme 1: BRP as selector (Fig. 1)

The interaction between antigen and antibody is used by way of example. It is possible to start with a cloned gene and derive an antibody-binding domain, or alternatively to start with an antibody and derive a DNA

15 sequence which encodes proteins which will bind to that antibody. In the first case, the cloned gene is expressed on the surface of bacteria within the context of the BRP and challenged with a library of antibodies expressed in the phage within the PRB. Only phage expressing an

20 antibody specificity able to bind protein epitopes encoded by the cloned gene will infect the selector bacteria and hence proliferate. In the second case, the expression of the cloned (and unknown) gene in the BRP on the bacterial cell surface is replaced by the expression of a suitable

25 form of cloned antibody. The library expressed in the PRB now consists of either random peptides or suitably selected cDNA sequences (e.g. a library of cDNA sequences derived from a tissue or cell which is known to express the protein recognised by the antibody). In principle, the two cases

30 are identical, and a discussion of the details of one of them is given.

For the selection of an antibody binding to the protein product of a cloned gene, the PRB is replaced with a fusion protein which contains an antibody domain (ScFv,

35 dAb, Fvs, Fabs, or derivatives thereof), as well as the normal PRB (see below for different forms of the fusion protein). The antibody domain expressed in each phage will

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be different, and hence have a different specificity. The
resulting phage library can be propagated on its normal
host using the original recognition specificity of the PRB
which should not be affected by the incorporation of the
antibody (domain). If the normal recognition specificity
of the PRB is lost by the incorporation of an antibody
domain, the library may be created and propagated by
relying on bacterial clones which express antibiotic
resistance encoded by a suitable phage. In this case,
phage can be isolated by growth of the bacteria (in liquid
culture, on solid media, or a combination of both) and
harvesting of supernatant, or by induction.

To select an antibody against the antigen X (the
protein product of the cloned gene X), a mutant BRP gene,
which has within its coding region a DNA sequence encoding
the antigen X (or part of it), is created. The antigen X
is expressed in a part of the BRP which is required for the
normal phage/bacterial infectious interaction. The result
is that the protein product of such a fusion gene (BRP-X)
will not be permissive for infection by the normal BRP/PRB
interaction. If BRP-X is expressed in a BRP⁻ (BRP
negative) bacterial host, the normal phage/bacterial
infection mechanism will be disrupted as the only bacterial
receptor for phage present (BRP-X) is unable to bind the
normal phage receptor for bacteria. If such a bacterial
cell is confronted with a phage antibody library, the only
way for infection, and hence phage (and phage antibody)
amplification, to occur is if an alternative recognition
system exists. The antigen X/anti-X antibody interaction
provides such a system and allows amplification of the
anti-X phage antibody. Phage antibodies with other
specificities will fail to be amplified. Thus, an antibody
recognising antigen X can be derived both as protein (as
phage antibody) and as a cloned DNA sequence after
confrontation of the phage library with the BRP-X-
expressing bacterial host.

In the event that the starting library is not complete
in its antigen recognition capability, with the result that
no antibodies, or ones of low specificity, are obtained, it
is a simple matter to immunise mice with the bacterial
5   strain expressing the recombinant BRP-X. The mice (which
may be made tolerant to the bacterial host) will mount an
immune response, providing a source for a new library
enriched for antibody specificities against X. In this
case too, it is clear that there is no need to purify the
10   antigen to immunise the mouse, as it is known that BRP-X
can act as a good vaccine.

An alternative method to improve (and even modify)
phage antibody specificities is by the creation of
hierarchical libraries, which involves taking an antibody
15   with a known affinity for an antigen and seeing if this
affinity can be changed by keeping one chain (e.g. the
heavy chain) fixed and varying the other in the form of a
library. This library of antibodies is then screened
against the antigen. Once a new antibody has been derived
20   (containing one original chain and a new one found in the
library), the process can be repeated, varying the chain
which was previously held constant.

The ingredients of this system are: PRB which retains
its wild-type specificity (this makes propagation easier,
25   although is not essential) as well as the new antibody
specificity which will form part of the antibody library;
and two bacterial strains, one wild type to propagate the
library, and one mutant strain in which the wild-type BRP
domain is substituted by the fusion protein BRP-X, and in
30   which infection can only occur via the interaction of
X/anti-X.

The BRP used on BRP-X need not be the same BRP used to
propagate the library, and does not even need to be the BRP
normally recognised by the PRB used by the virus. It may
35   be a BRP used by another virus which may or may not be
related. It is important only that after PRB-(anti-X) has
bound to BRP-X that transfer of the phage genetic material

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passes into the bacteria expressing BRP-X. The use of techniques such as electroporation or low salts, may allow this to occur in cases in which the choice of PRB-(anti-X) and BRP-X allows binding but not infection.

Selection can occur either in liquid phase, in agar or in a combination of liquid followed by agar. In liquid culture, one expects the overgrowth or survival of the phage expressing the desired binding activity. In agar, plaque formation (or the formation of resistant colonies) reflects the successful interaction between phage and bacteria.

The system can, in principle, exploit different bacteriophage/bacterial host interactions, such as the coliphages lambda, M13 (f1, fd), T even phages, T odd phages, RNA phages or even phages of other microorganisms. By way of example, the procedure may be used to engineer this system first in the lambda/lamB system, secondly in the fd/sex pilus system, and thirdly using a mixed system. It is clear that equivalent manipulations may be made in PRB and BRP pairs as described above (T4 gene 37 protein/ompC, T2 gene 38 protein/ompF), and any others, as well as the artificial modification of one of the pairs so that the host range is changed, or the creation or engineering of novel BRP or PRB.

A demonstration that the procedure is able to function can be provided by the creation and use of an antibody of known specificity. Y13.259 is a monoclonal antibody which recognises a 17 amino-acid epitope found within the p21ras molecule. The manner in which the expression of the variable regions of this antibody as ScFv, FAb or Fv as part of the J protein, and the expression of the corresponding epitope within the lamB protein, is as follows:

Construction of anti-ras (anti-X) lambda antibodies

Strains: DH5αF' was used for all general cloning, Pop6510 for expression of all lamB derivatives, and LE392 for amplification of lambda.

Except where otherwise stated, all methods used are as described in "Molecular Cloning: A Laboratory Manual" (1989) Cold Spring Harbour Laboratory Press. Vector construction: ScFv, FAb or Fv forms of Y13.259 (see Werge

5   et al (1990) FEBS Lett. 274 193-198), under the control of a promoter, are constructed by taking the isolated heavy and light chains and inserting them into vectors which allow expression of the appropriate antibody form. This done by standard cloning techniques, PCR, or PCR-induced

10   ligation techniques. Such vectors are widely available and the important elements include: a bacterial promoter (e.g. LacZ), a ribosome-binding site, an appropriate bacterial leader sequence (e.g. pelB, ompA), stop codons at the ends of chains, and an appropriate linker sequence between heavy

15   and light chains. For ScFv, such a linker sequence would encode a peptide linker which would connect VH to VL, e.g. [gly$_4$ser]$_3$ or the cellulase linker; for Fv, the DNA linker sequence encodes a stop codon at the end of the upstream variable region, a ribosome binding site, and a bacterial

20   leader sequence; for FAb, the variable regions are attached to the CH1 and CL1 constant regions and separated by linkers similar to those found in Fv. A protein tag (e.g. the myc tag) to detect the cloned antibody form is useful, but is not essential. The order of the chains has been

25   shown not to be important in other antibodies, and would be expected not to be important in this case. Other variations on the normal immunoglobulin architecture are easily envisaged (e.g. ScFv attached to light chain constant regions, or heavy chain constant regions lacking

30   CH1). The particulars are not as important as the creation of a cloned antibody form which is able to bind the peptide described. This can be confirmed by Western Blotting, ELISA, binding to a column or any other technique used to detect antigen/antibody binding. ScFv Y13.259 has been

35   produced and shown to bind its epitope.

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Once the specificity is confirmed, the DNA encoding
the antibody is cloned in frame to a fragment of the J gene
of bacteriophage lambda in a suitable position which had
been separately cloned from lambda into a general purpose
5   vector (such as pUC19 or Bluescript).

One important feature of the J gene antibody fusion
constructs is that, by way of example, the BstBI site in J
(equivalent to nucleotide 18048 in lambda) is retained
within the plasmid construct. This is found towards the 3'
10   end of the gene. The genetic evidence (see Fuerst et al,
supra) suggests that it separates the host-determining
region from the structural component, and so allows one to
manipulate the host-determining region of the J gene in a
plasmid rather than in lambda. J gene fusions can
15   therefore be made which are then easily cloned back into
lambda using BstBI to join the structural part of the
normal J gene found in the lambda donor left arm to the
recombinant J genes produced in the plasmid. An XbaI site
at the 5' or 3' end of the fusion construct serves to clone
20   the J gene fusion construct back into lambda by providing
a site at the 3' end of the J gene.

These sites are convenient, but any other sites which
serve similar functions could be used, and even engineered
sites (e.g. rare cutting sites such as NotI or SfiI) could
25   be included within the destination lambda vector as well as
the J/Ab fusion vector. Sites further 5' of BstBI (e.g.
SnaI, KpnI with remaining sites in J removed) could be used
instead of BstBI if a greater part of the J gene is found
to be required.

30   These sites can be used for cloning the J/Ab fusion
construct into lambda as follows:

1.   Purification of arms: lambda left arm cut with BstBI,
and lambda right arm cut with XbaI would be separately
prepared and purified (on a gel, or by sucrose density
35   centrifugation in the presence of oligonucleotides
complementary to the cohesive end of the arm not required).
The source of the lambda arms will determine the phenotype

of the esulting recombinant phage. The use of temperature sensitive repressor (cI857) will allow the harvesting of phage after lysogeny by temperature induction.

2.   Cloning: equimolar amounts of the arms are mixed with
5   equivalent amounts of the J/Ab fusion construct cut with XbaI and BstBI.   In the case where the XbaI site is directly downstream of J/Ab, the resultant recombinant lambda clone will retain all essential genes, except that the normal J gene is replaced by the J/Ab fusion gene. If
10   the XbaI site is upstream of J/Ab, the resultant recombinant lambda clone will be identical to that above except that the gene for amp resistance (as well as the other plasmid genes) will be also included. Other plasmids containing other resistance markers (e.g. tetracycline,
15   kanamycin or chloramphenicol) may also be used.

3.   Packaging: after in vitro packaging and infection into appropriate cells, plaques can be picked, or if the amp resistance has been retained, amp resistant colonies can be picked after plating on low concentration ampicillin plates
20   (20-30 µg/ml) and growing at 30°C. The use of hfl (high frequency of lysogenisation) lon protease negative bacterial strains would be expected to increase the frequency of amp resistance colonies and stabilise the fusion protein. Phage can be isolated from the colonies by
25   heat induction (to inactivate the cI857 temperature sensitive repressor) or just by growth of the lysogenic bacteria, since phage are released naturally into the culture medium, even at lowered temperatures. The use of lowered temperatures may be important for the stability of
30   the J/Ab fusion protein, as it is when antibodies (as ScFv or Fab) are grown in bacteria.

Alternative forms of lambda antibody constructions (Fig. 4)

        Alternative forms of the J/Ab fusion proteins include the following:

35   1.   Instead of using wild-type lambda as the source of the J gene, that part of the J gene responsible for the host range phenotype of strains λho, λhh* and K10 (between bases

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18048 and 18890 of lambda) can be used, replacing the wild-
type sequence in this position. This may be done using PCR
to amplify the corresponding segment of DNA, and standard
cloning.   These different host range mutants may be
5   differentially sensitive to the presence of the Ab attached
to their C terminal end or within the J gene protein.

2.   Different antibody forms: deletion of part of the
antibody (done using PCR and cloning) may be useful to
orientate it in a different direction if there is steric
10   hindrance of J with respect to Ab or vice versa.   The
removal of a single ß strand (e.g. amino-acids L1-14 or
H1-13) would be expected to change the orientation of the
antibody without affecting its binding specificity. It is
clear that any derivative of any antibody or binding
15   molecule can be used.   The use of antibody forms which
require two separately synthesised chains (FAb, Fv etc) may
have one of the two chains synthesised as a soluble
intracytoplasmic derivative. This be expected to associate
with the other chain covalently linked to J as a fusion
20   protein.   In this case bacterial leader sequences would be
deleted.

3.   The region of the J gene coding for host range
specificity (and hence the PRB specificity) may be
completely deleted and replaced with any of the antibody
25   forms described.   In this case, propagation of the lambda
phage occurs in a lysogenic state (after in vitro
packaging, infection and selection for antibiotic
resistance), but it is expected that successful infection
will occur if the BRP (lamB) contains an appropriate
30   antigen (lamB-ras) for the PRB-Ab (J-Y13-259).

4.   A linker can also be introduced between the Ab and the
J protein.   Such a linker may be one found naturally
between two protein domains. Argos, J. Mol. Biol. (1990)
211: 943-958, discuss short linkers which can be used, and
35   Takkinen et al, Prot. Eng. (1991) 4 (7): 837-841, describe
longer linkers. Alternatively, an artificial linker may be
created by the use of contiguous short linkers. A linker

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may also be found experimentally by the creation of a random linker library between the two domains. This may be done by PCR using appropriate oligonucleotides. This should provide flexibility between the two protein domains without being susceptible to proteolysis, and may overcome steric hindrance.

5.   The antibody may be put at a number of different positions within the J gene. One position to insert the antibody is at the 3' end of the J gene (the C terminal end of the protein). Sequencing of that part of the J gene responsible for the host range phenotype of strains λh+, λho, λhh* and K10 (between bases 18048 and 18890 of lambda), may reveal differences between these strains which are responsible for the host range differences. Regions of particular diversity between the strains may be responsible for the host range differences and may better tolerate the insertion of the antibody.

6.   In addition to the use of a J/Ab fusion gene, a normal J gene may be provided by a lysogenic heteroimmune phage, or on a plasmid. This allows the creation of a hybrid phage which contained a normal J protein which can be used for propagation in normal hosts, and the recombinant J/Ab protein which can be used for selection. This is similar to the manner in which helper phage provides normal gene III protein in the pHEN1 system; see Hoogenboom et al, Nuc. Acids. Res. (1991) 19: 4133-4137.

7.   Any one, or a combination, of the above modifications can be used (e.g. Ab within the J gene containing a different host range mutation).

Construction of lamB/ras (BRP-X)

To test the lambda Ab described above, a suitable bacterial host expressing p21ras within lamB on the outer surface of an E. coli which otherwise lacks lamB is required. This may be done by as described by Werge et al, Oncogene (1992) in press, cloning the epitope of p21ras recognised by Y13-259 Ab, itself produced by the hybridisation and phosphorylation of complementary

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oligonucleotides which encode residues 56-76 of p21ras into
the BamH I sites of lamB in vectors AJC352, AJC264 and
AJC178; see Charbit et al (1988), supra. The hybridised
oligonucleotides have BamH I complementary ends (GATC) at
5   both ends, but only at the 3' end would the BamH I site be
recreated. This facilitates mapping. Plasmids containing
modified lamB genes may be transfected into pop6510, and
bacterial clones containing the p21ras epitope in correct
orientations may be tested for their ability to bind anti-
10   LamB serum as well as both native Y13-259, and its
derivative forms, by ELISA on intact cells, as well as by
western blotting.

Procedure for the demonstration of infectious lambda
antibodies

15   Phage prepared by amplification in LE392 or other
cells (either by lysis or heat induction of lysogeny) may
be tested for their ability to bind the p21ras antigen (by
e.g. enrichment of phage on a p21ras column) after testing
the integrity of the J/Ab fusion protein (by e.g. western
20   blotting), and then for their ability to infect pop6510
cells expressing p21ras by infection of a mid log culture
of pop6510 lamB/p21ras and plating of dilutions. The
synthesis of the lamB/p21ras may be induced with IPTG in
the system described (lamB synthesis is under the control
25   of the tac promoter in AJC plasmids). That the infection
occurred by the expected peptide/antibody interaction would
be shown by the need for the epitope within lamB to allow
infection to occur. That it was possible to recover the
Y13-259 lambda antibody from a library would be shown by
30   making various dilutions of the Y13-259 lambda antibody
into a library of diverse lambda antibodies created as
described below.

Construction and amplification of a lambda antibody library

Once infection by an interaction different from that
35   of the normal PRB/BRP mechanism has been shown to be
feasible, a library of variable regions (VH and VK) may be
cloned into the same sites of the J gene. This may be

20

carried out using a similar method to that described by
Marks et al (1991), supra except that, after assembly and
amplification of the Ab form used, it is cloned into lambda
arms cut with appropriate sites (e.g. if the same
5   oligonucleotides were used: SfiI and NotI engineered within
the lambda for the purpose).

The recombinant lambda vector encoding the lambda
antibody library may be in vitro packaged and infected into
LE392 for amplification. The library of lambda antibodies
10   (as phage articles) may be harvested after amplification in
liquid (or plate) culture using standard methods (lysis or
heat induction).

Procedure for the selection of lambda antibodies

DNA sequences encoding epitopes (X) from the gene for
15   which an antibody is to be derived are cloned into the lamB
gene in frame in the appropriate site using either
oligonucleotides, cloned pieces of DNA, or DNA obtained by
PCR amplification. The presence of a BamH I cohesive end
at each end is required. The recombinant lamB/X clone is
20   transfected into a cell line lacking lamB (pop6510) and the
bacterial clone cultured.

Selection of appropriate lambda antibody specificities
involves a confrontation between the lambda antibody
library and the bacterial clone expressing lamB/X. This
25   involves inoculation of a culture of the bacterial clone
culture with an aliquot of the lambda antibody library.
After culture, the specific lambda antibody is isolated by
plating onto a lawn of the recombinant bacteria (pop6510
lamB/X). Plaques are picked, amplified, and tested for
30   their ability to infect pop6510 lamB/X at a much higher
efficiency than the complete library, as well as an
inability to infect pop6510 lamB/Y, where Y is a different
specificity.

If lambda with a temperature-sensitive mutation in cI
35   (e.g. cI857) is used, and development of plaques occurs at
the permissive temperature (32°C), they will be turbid.
This allows the growth of lysogenic bacteria which can be

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induced to produce phage upon heat induction. Alternatively, the use of a selective marker and hfl bacteria (as described above for the cloning procedure of lambda antibodies) can be used to select resistant colonies from which lambda and bacteria can be isolated.

Further tests of the specificity of the lambda antibody (as a lambda phage particle) may involve ELISA, immunoprecipitation and western blotting on pop6510 lamB/X bacterial cells, using appropriate non-X-expressing bacterial strains as controls. Measurement of the affinity of the lambda antibody for its bacterial target can also be done using dilution analysis. Since purified natural antigen is not available, further tests of the lambda antibody specificity on native material are required. This can be done using ELISA, immunoprecipitation or western blotting on appropriate extracts.

The detection of lambda antibody binding can be performed using secondary antibodies against endogenous lambda proteins or tags present on the ScFv/J protein fusion protein.

Downstream use of lambda antibodies

Lambda antibodies can either be used as phage particles containing antibody specificities, or the heavy and light chain variable regions can be cloned out using standard procedures and inserted into any one of a number of compatible vectors which allow expression of protein in a wide variety of different cellular systems, including prokaryotes, eukaryotes and plants. When cloned into the appropriate vectors the antibody specificity can be coupled to desired effector functions, a review of which is given by Winter et al, Nature (1991) 349: 293-299.

The fd (f1/M13) sex pilus system

In lambda the PRB and the BRP are well recognised. This is not the case in fd (which in the following discussion will be used interchangably with f1 and M13, these being three phages which are essentially identical, but have been used in different studies), where the PRB is

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well recognised, but the identity of the BRP is less clear.
There is little doubt that the BRP resides within the sex
pilus, but its molecular nature is less clear (it is
possibly f pilin). However, definitive identification of
5 the BRP for fd can be done in a number of standard ways (as
described widely for many receptors in the literature).
However, scheme 1 may also be used to identify the BRP for
fd.

Deletion mutants of gene III previously have revealed
10 that the N terminal region is important in binding to the
sex pilus and infection. Under certain conditions, M13 is
able to infect F⁻ (lacking the F pilus) bacteria, a region
required for this activity having been delimited to the N
terminal 99 amino-acids. Deletion mutants of CAT lacking
15 the complete N terminal region (aas 1-196) or just part of
it (aas 99-196) should not be able to infect bacteria by
the normal route. The latter, but not the former, would be
expected to be able to infect F⁻ bacteria, and both would
be expected to be able to contain antibodies as a fusion
20 protein with the gene III protein.

Immunisation of mice with either purified pili, or
whole F' bacteria, has been shown to yield an antiserum
able to inhibit infection of F' bacterial cells with fd.
The creation of a library of fd phage antibodies from the
25 spleen of a mouse immunised in this fashion should yield
some phage antibody particles which are able to recognise
the normal binding site of fd. Some of these phage
antibodies would be expected to be able to infect F' cells
using as PRB the antibody rather than the normal PRB (which
30 has been deleted). The identification of a phage antibody
of this type would be the first step towards the molecular
identification of the BRP.

The characterisation of a protein which is recognised
by a monoclonal antibody is a common and relatively
35 straightforward procedure, and may be conducted using
standard techniques. Once characterised, insertion mutants
are created by the insertion of restriction enzyme sites,

either in a random fashion as has been done with lamB, or
in a more directed fashion using PCR. and prediction of
secondary structure (targeting the insertions to those
parts of the protein expected to be exposed) is relatively

5   straightforward. The insertion of restriction enzyme sites
at strategic positions allows an investigation of the ffect
on fd infection, and on the effect of the insertion of
extraneous protein. In this manner, a system may be
created, in a manner similar to that created for lamB (with

10   the development of appropriate bacterial mutants lacking
the BRP, and plasmids expressing the deleted BRP on a
plasmid with a selectable marker), with the implementation
of the four schemes described above.

Mixed Systems (Combination of lamB and fd systems):

15   There is an alternative manner in which a scheme
similar to scheme 1 may be operated. This involves cloning
of an epitope into lamB, and using the bacteria as a solid
phase selector to screen a library of phage antibodies.
(which may have been derived from a mouse immunised with

20   the same recombinant bacteria). The advantage of the use
of recombinant bacteria as a solid phase selector (a
"living column") to select phage antibodies is the same as
that described for scheme 1 above: an antibody can be
derived against the protein product encoded by a DNA

25   sequence just be cloning it into the appropriate vector,
without the need to produce or purify the protein.

The library of phage antibodies is precleared with
identical bacteria not expressing the recombinant epitope,
before using the recombinant bacteria as a selector. After

30   allowing binding to occur, the phage antibody particles are
eluted (after washing and resuspending the bacteria) with,
e.g. 100 mM triethylamine or a low pH glycine buffer, the
column bacteria which could compete are removed (by
centrifugation), and an aliquot of the supernatant

35   containing the phage antibody particles to bacteria able to
support the infection of fd phage (TG1, DH5αF', JM101 etc)
is added. Theoretically, if the lamB/X fusion protein is

expressed in such bacteria, elution and infection could occur in the same bacteria, by just allowing reexpression of the F' pilus. In this case, the individual bacteria expressing the antigen X will not necessarily be infected by the same phage which bind to them. For this reason scheme 3a, as described, is not possible, as individually interacting members will not be co-isolated, although it will be possible to isolate a polyclonal phage stock which will recognise a number of different bacteria expressing different recombinant lamB/X on their surface. It is clear that the use of the living column does not require either of the binding partners to be expressed in the BRP or PRB. Any protein expressed on the surface of either bacteria or phage would be suitable.

Infection by other means

It is known that fd can infect F$^-$ bacteria at an efficiency 10^{-6} of that when infecting F' bacteria, suggesting that infection can occur independently of the normal mechanism. A modification of the living column described above could be used to infect F$^-$ bacteria. This involves binding of phage antibody to bacteria expressing lamB-X (after preclearing the phage antibody library against bacteria not expressing lamB-X), and then plating directly (with or without treatment such as electroporation, low salt solutions as used to make competent cells, or enzyme, e.g. mild lysozyme). Resistant bacteria would be expected to arise from the binding interaction. This is an example of the use of non-homologous BRP/PRB pairs.

Combination of fd and N

fd and IKe are similar filamentous phages with a overall homology of 55%. They recognise different BRPs (fd recognises the F' pilus, and IKe recognises pili encoded by N incompatibility group plasmids). However, their structure is sufficiently similar that the transfer of the PRB from one to the other should allow infection via the BRP recognised by the transferred PRB to occur.

25

It is known that antibodies can be placed within the PRB of fd. It would be expected that the insertion of antibodies (within the PRB of fd) which recognise the BRO encoded by the N incompatibility group plasmid would allow

5   infection of that host. Furthermore, if the BRP (encoded by the N incompatibility group plasmid) was engineered to contain an epitope recognised by an antibody, infection should occur if that antibody was found within the PRB of fd. Manipulation of the BRP encoded by the N

10   incompatibility group plasmids would have to be performed as described above for the BRP for fd.

This is another example of how the implementation of scheme 1 would allow the identification of a useful protein (the BRP encoded by the N incompatibility group plasmids).

15   Alternative uses of Scheme 1

In addition to the selection of antibodies, or fragments thereof, from libraries of such molecules expressed on the surface of phage, this novel method may be used to select peptides or proteins which are able to bind

20   other peptides or proteins. Although described for lamB, the examples described below could also be carried out with the fd/sex pilus, and fd/lamB systems, any other PRB/BRP combination, or any pair of proteins exposed on the surface of bacteria and phage as described in the "living column"

25   as above.

1.   Agonists or antagonists of growth factors, hormones, lymphokines or any other bioactive molecules. E.g. the expression of a receptor (or part thereof) for, or an antibody recognising, such a molecule within the lamB

30   protein would allow selection of a binding protein or peptide from a random library of such molecules. The effect of the selected molecule on the biological system under study could then be tested.

2.   Molecules capable of inhibiting the function of

35   (pathogenic) viruses or bacteria. E.g. expression of CD4 (or part thereof), or the envelope protein (or part thereof) of HIV (the AIDS virus), within lamB would allow

selection of a protein or peptide which may be able to inhibit the interaction of HIV with CD4 (its normal receptor).

3. Binding molecules, other than antibodies, useful in diagnostic procedures. E.g. the expression of tissue specific molecules within lamB would allow the selection of molecules which would have tissue specific binding by virtue of their selected phenotype.

4. Inhibitors or activators of the function of any protein whatsoever. E.g. the expression of an enzyme (or part thereof) within lamB would allow the selection of molecules which would be able to bind that enzyme. Further testing would reveal whether the molecues selected enhanced or inhibited the activity of the enzyme. Such effects would be expected to occur by the induction or stabilisation of conformational changes upon binding, by steric hindrance, by the inhibition of substrate binding or product release, or other mechanisms.

5. Novel enzymes with new or enhanced activities. The expression of reaction intermediate, or a molecule resembling such, within lamB would allow the selection of a protein capable of binding such a molecule. By careful choice of the form of the selected element (e.g. based on known canonical enzyme structures such as the TIM barrel, or catalytic antibodies), enzyme activities may be selected for.

As for phage antibodies, above, selected molecules may be used after they have cloned out of the J gene of lambda, or they may be used as lambda particles with specific binding properties or activities. In the former case they may be expressed as proteins and purified as such, or they may be expressed within cells, transgenic animals or plants, as individual proteins or as fusion proteins.

Scheme 2: PRB as selector (Fig. 2)

The creation of antibody libraries will again be used to illustrate the methodology of scheme 2, bearing in mind that this scheme too, is applicable to any interaction

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between two protein species. Although the methods are described for lambda and lamB, any of the other phages described above could also be used.

The modified lambda DNA, carrying the J gene/peptide
5   (antigen) fusion protein, is produced in a fashion similar to that described in scheme 1, replacing the ScFv described with DNA coding for the appropriate peptide or antigen. The DNA coding for the peptide is obtained by cloning, by PCR amplification using appropriate oligonucleotides, or by
10   the use of oligonucleotides themselves to encode the desired amino-acid sequence. Appropriate sites have to be engineered into the ends of the oligonucleotides to allow cloning into appropriate sites. Best sites will be those not found, or found infrequently, within lambda. SpeI is
15   such a site which can be incorporated at the 3' end of the J gene (and hence at the 5' end of the oligo encoding the peptide) without affecting J gene function. SacI is a site which can be used for the 3' end of the oligo encoding the peptide, as it is found within lambda in non-essential
20   regions and so can be easily eliminated. A stop codon also has to be placed at the 3' end of the peptide sequence, if this is placed at the 3' end of the J gene.

Antibody variable region domains (or other protein species with binding activity) can be inserted within the
25   coding region of lamB. In a usual antibody variable region domain comprising heavy and light chains, the N terminal ends of the heavy and light chains are far, in molecular terms, from their corresponding C terminals. As this may impose structural constraints upon the incorporation of
30   variable region domains within the lamB protein, the removal of the first and last β pleated sheet from heavy and light chains should result in a compact variable region domain, the ends of which are very close together. This may help overcome the present size constraint of 70 amino-
35   acids as the longest peptide successfully inserted into lamB.

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It is envisaged that single VH domains or ScFvs can also be inserted into lamB. In the first case, soluble VL domains may also be expressed by the same cell into the periplasmic space to provide the necessary complement to
5   create antigen binding activity.

A library of such molecules may be created in a fashion similar to that used for lambda phage described in scheme 1 above. The difference is that, instead of cloning into the J gene of lambda, the library of specificities
10   would be cloned into the lamB gene within appropriately engineered sites in vectors AJC178, AJC264 or IC352. If soluble VL regions are used these would be encoded on a separate plasmid, or as a separate translation unit within the same plasmid. After transfection into e.g. pop6510,
15   the library would be amplified and used for selection by phage.

In this scheme the interaction between phage and bacteria must result in survival of the bacteria to allow amplification of the element (ScFv, Fv or dAb in this
20   example), selected by the selector found within the phage PRB (peptide antigen in this example). This can be done if a gene for a selectable marker (e.g. chloramphenicol, kanamycin resistance) is incorporated into the lambda vector under the control of a bacterial promoter. It is
25   important that this resistance marker is different to that used for AJC264 (ampicillin), and found naturally within pop6510 (tetracycline), if this plasmid/bacterial strain combination is used. After infection, the growth of resistant bacterial colonies will be induced. A lysogenic
30   strain of lambda, or one with a temperature sensitive mutation in cI (e.g. cI857), must be used in this system to prevent lysis of infected bacterial cells. In the latter case, colonies must be grown at 32°C, in order to preserve the activity of the lambda repressor and hence maintain
35   lysogeny. The use of hfl (high frequency of lysogenisation) negative bacterial strains may increase the frequency of resistant colonies.

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Bacterial colonies thus selected can be grown and manipulated in the normal fashion (albeit at lower temperature). The selected element can be identified following preparation and analysis (including DNA aquencing) of plasmid DNA. As for phage antibodies, above, selected molecules may be used after they have been cloned out of the lamB gene, or they may be used as bacterial particles with specific binding properties. In the former case they may be expressed as proteins and purified as such, or they may be expressed within cells, transgenic animals or plants, as individual proteins or as fusion proteins.

Alternative uses of scheme 2

Scheme 2 may be used in exactly the same ways as scheme 1, a number of examples of which have been described above.

The decision whether to use scheme 1 or scheme 2 for a particular purpose will probably depend upon the nature of the selector and the selected element. It is expected that, in the J gene, for example, the size of inserted DNA sequences coding for selector or selected elements will be limited. The same is known to be true for lamB, however the experimental limits of the two systems have not yet been clearly defined. Given the apparent symmetry of schemes 1 and 2, it is clear that, all other things being equal, it would be advantageous to place the larger molecule (selector or selected element) within the protein (lamB or J) which has the greater "capacity". Another factor which should be taken into account is the stability of the fusion protein produced. Some proteins may be more stable in a system which involves a secretion step (lamB), whereas others may be more stable when expressed as part of a "cytoplasmic protein" - as lambda proteins can be considered to be, since they are synthesised intracellularly before the bacteria lyses to release the phage.

Another alternative reason for picking one system over another would be the final form of the selected element; i.e. selected elements expressed on phage may be more useful in some circumstances than if they were expressed on

5   bacteria.

Scheme 3: confrontation of libraries of specificities - the selection of couples (Fig. 3a)

In this scheme, two libraries of specificities, one on phage and one on bacteria (within the PRB and BRP,

10   respectively) are confronted. Use of the terms selector and selected element is less valid in this case, as selection is not for a specific binding activity, but for an interaction which leads to infection of the bacteria by the phage. Instead, there are two libraries (A and B),

15   individual elements of which (species A and species B) may be able to interact with one another. Within species A and species B, there are (for the purposes of the present invention at least) selectors and potential binding partners, and the terms "selector" and "binding partner"

20   must each be understood to include a plurality of species.

Scheme 3a is operated in a fashion similar to that of scheme 2: i.e. the successful encounter of phage and bacteria will lead to bacterial colonies (rather than plaques) which will represent the productive binding of one

25   element of species A with one of species B. As in scheme 2, the selection of colonies following interaction will depend upon the inclusion of a selectable marker within the lambda genome. Although the description below refers to lambda/lamB, any other BRP/PRB system could be used.

30   Rescue of selected elements

The rescue of the selected element cloned into the lamB gene will occur in a fashion similar to that described above in scheme 2, i.e. growth and amplification of the selected bacterial colony and analysis of the plasmid DNA

35   it contains, and the lambda it is able to produce.

The rescue of the selected element cloned into the J gene involves induction of the lysogenic bacterial colony.

If the lambda vector contains a temperature sensitive
mutant of the cI repressor (e.g. cI857), phage can be
isolated from the colonies by heat induction (to inactivate
the cI857 temperature sensitive repressor) or just by
growth of the lysogenic bacteria, since phage are released
naturally into the culture medium, even at lowered
temperatures. The use of lowered temperatures may be
important for the stability of the J/Ab fusion protein, as
it is when antibodies (as ScFv or Fab) are grown in
bacteria. The use of hfl bacterial strains may increase
the frequency of amp resistance colonies; this may be
important when screening a large number of possible binding
interactions, and the use of lon protease negative bacteria
may stabilise the fusion protein. Lambda, and DNA derived
from it, can then be isolated from the supernatant using
standard procedures.

As for schemes 1 and 2, above, selected molecules may
be used after they have been cloned out of the lamB gene,
or the J gene. They may be expressed as proteins and
purified as such, or they may be expressed within cells,
transgenic animals or plants, as individual proteins or as
fusion proteins. Interacting pairs of molecules isolated
in this way may also be studied in situ as phage/bacterial
pairs, to determine, for example, affinity constants,
interactions with other molecules and stability.

Uses of scheme 3

The selection of an interacting pair rather than an
individual selected element is likely to prove very useful
under a number of different conditions. Antibody/antigen
interaction is again used for the purpose of illustration.
1. The gene or cDNA coding for a protein of unknown
function can be used to produce a monoclonal phage (or
bacterial) antibody, using schemes 1 or 2 above. However,
by the use of scheme 3, this powerful technique can be
extended. Instead of the use of a single selector to
isolate the antibody, a library of fragments derived from
the cDNA or gene can be used. These can be isolated after

appropriate restriction digestion or PCR amplification. After library confrontation, phage (or bacterial) antibodies binding to different parts of the cDNA or gene used will be produced. This will yield not only a

5 "polyclonal phage (or bacterial) antibody" directed against different epitopes which can be used as a mixture, but also a rich source of monoclonal phage (or bacterial) antibodies which can be used individually.

2. The study of structure-function relationships between

10 antigen/antibody, or indeed any other protein/protein interaction is greatly facilitated. An antibody against a protein antigen X may be randomly mutated, to produce a library of anti-X antibodies. This library then may be confronted with a similar library of mutants of the antigen

15 X. Mutations in either antibody or antigen which inhibited the normal interaction of anti-X with X, could only be selected for by the binding to partners with compensatory mutations. In this way one may select pairs of anti-X/X which were able to bind to one another but not to the

20 unmutated partner. By the sequencing of such pairs one would obtain a greater understanding of the molecular nature of the interaction of X with anti-X, without the need for X ray crystallography. Examples of other protein interactions which could be studied using this method

25 include: transcriptional activator/repressor pairs, proteins involved in the uptake of viruses by cells (e.g. CD4, HIV envelope protein), individual elements in multi-protein or enzyme complexes.

3. The creation of "polyclonal phage (or bacterial)

30 antibodies" against the protein product of a cDNA or gene described above can be extended to allow the selection of phage or bacterial antibodies against any coding sequence at random. This is likely to prove very useful and important in situations in which large amounts of DNA

35 sequence data are acquired at random. This is the case in the projects underway to sequence a number of different genomes (such as E. coli, yeast, C. elegans, Drosophila,

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mouse and human). The identification of potential coding
sequences can be undertaken using computer analysis or exon
trapping via retroviral intermediates; these can then be
isolated en masse using PCR amplification with appropriate
oligonucleotides.   A library of such random coding
sequences can be used to select from a library of random
antibodies (or indeed any binding proteins) those which
bind. The isolation of bacteria/phage pairs will give, in
one step, antibodies against the protein products of
defined DNA sequences.

4.   The accumulation and analysis of DNA sequence data has
revealed that many proteins have counterparts encoded by
similar genes. In many cases the assignment of the gene
encoding a particular protein to a family of genes is made
on the basis of the conservation of certain sequence motifs
(e.g. immunoglobulin superfamily, family of tyrosine
kinases, family of DNA binding proteins such as
homeodomains or zinc finger-like domains)).   This
conservation of sequence motif has been used to isolate
other members of the same family by either standard library
screening using DNA hybridisation, or by PCR amplification
using the conserved sequences to design the amplifying
oligonucleotides employed. It is clear that this use of
PCR amplification of related genes can be used in scheme 3.
Such related genes can be cloned into the lamB gene using
oligonucletides corresponding to conserved regions of these
gene families. This will create a library of coding
fragments of related genes which can be used to derive
phage antibodies, or binding peptides, which will have a
range of specificities, from specific for individual
members of the gene family, to general specificities which
recognise all members of the family.

5.   If both libraries A and B consist of antibodies, the
interactions which will be selected for will be of the
nature: idiotype/anti-idiotype. The isolation of a large
number of these will prove to be useful in immunological
studies as well as in future antibody engineering.

In cases 2, 3 and 4 above, the likelihood that useful antibodies are selected can be increased by using the library of bacteria (expressing the fragments of coding sequences within lamB) to immunise mice, and then to use the spleen of such mice to create the library of antibody specificities.

It is important to note that in scheme 3 confrontations of libraries can occur with no knowledge of the precise DNA sequence of the elements A and B. This will occur, for example, if mutations (created at random) in known sequences are used to create the libraries, or unknown sequences derived following PCR amplification. The precise sequence of the interacting elements will not be known until after they have interacted, and then been isolated and analysed.

Scheme 3b: confrontation of libraries of specificities - the selection of singles (Fig. 3b)

An alternative to the selection of couples of interacting elements, as described above, is the selection of single non-interacting lements from a pool of interacting ones. Also in this scheme two libraries of specificities, one on bacteria and one on phage, will be confronted. Although, in principle, the libraries do not need to a restricted or preselected to allow selection of singles, it is clear that to fully exploit this scheme the two libraries should be reselected, according to any of the schemes above. This is because non-selected libraries will contain many spurious singles. Preselection of libraries will ensure that the selection of singles occurs from within a population with a predetermined pattern of specificities.

Rescue of selected singles

Two cases are to be distinguished: the selection of phage not interacting with any elements in the library expressed within the BRP, and the selection of bacteria not interacting with any elements in the library expressed within the PRB.

35

Selection of bacterial singles

    To select non-interacting bacteria from a library of
bacteria expressing different specificities within their
BRP upon challenge with a library of phage expressing
5   different specificities within their PRB, it is only
necessary to perform the confrontation under lytic
conditions. All bacteria which can be recognised by phage
will be lysed, and the only ones resistant to lysis will be
those expressing a specificity not recognised by an element
10 within the phage library. Surviving bacteria can be
amplified and rescued.

Selection of phage singles

    There are two ways in which non-interacting phage from
a library of phage expressing different specificities
15 within their PRB upon challenge with a library of bacteria
expressing different specificities within their BRP can be
selected:

a)   Absorption of the phage library upon the bacterial
library under condition in which infection is prevented.
20 b)   Infection of the bacterial library with the phage
library under strictly lysogenic conditions. Interacting
phage will disappear as phage particle as they are taken up
by binding bacteria.

    Phage particles will be able to be rescued and
25 amplified using standard methods from a supernatant in both
cases.

Application of scheme 3b in the analysis of human genetic
disease

    The use of scheme 3b to derive specific probes (DNA,
30 antibody or binding proteins or peptides) for modifications
at genetic loci, such as those found in many genetic
diseases, is readily envisaged. This will involve the
combination of scheme 3b with schemes 1 and 2 above. An
example ia the identification by RFLP, or other genetic
35 methods, of the linkage between a certain human genetic
disease and a region of DNA. The resolution of genetic
techniques is usually of the order of hundreds of

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kilobases. The cloning of a library of coding regions from
this chromosomal locus from both normal (termed the N
epitope library) and mutated (termed the M epitope library)
forms of the locus into lamB could provide libraries of
5   epitopes from which one may select:
a)   specific phage antibodies to the unknown mutated
epitopes.
b)   specific phage antibodies to the normal versions of
the mutated epitopes.
10   c)   candidates for the unknown mutated epitope, or
d)   in the case of mutations which involve deletions,
candidates for the normal epitope lost or affected by the
disease mutation.
     By way of example, after challenge of the N epitope
15   library with a phage antibody library (either general or
derived following immunisation with bacteria expressing the
N epitope library) using scheme 3, infectious phage
antibodies (termed the N restricted phage antibody library)
harvested may be used to:
20   a)   Infect the M epitope library under conditions allowing
bacterial lysis. Surviving bacteria carry epitopes not
recognised by the N restricted phage antibody library and
therefore potential candidates for (or an epitope of) the
mutated locus.
25   b)   Absorb the N restricted phage antibody library on an
excess of the M epitope library (under conditions where
infection will not occur). Phages failing to be absorbed
will be candidate phage antibodies for the normal version
of the mutated epitope.
30   c)   An alternative to the use of absorption to derive
candidate phage antibodies for the normal version of the
mutated epitope is to perform the challenge of the N phage
antibody library with the M epitope library under stringent
lysogenic conditions. Under these conditions, the only
35   phage surviving as phage particles will be candidates for
those recognising the normal version of the mutated locus.

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The scheme can and should be reversed using M phage antibody libraries (derived by challenging the M epitope library with a phage antibody library). This M phage antibody library could then used to:

5   a)   Infect the N epitope library under conditions allowing bacterial lysis. Surviving bacteria would carry epitopes not recognised by the M phage antibody library and would therefore be potential candidates for (or an epitope of) the normal version of the mutated locus.

10   b)   Absorb the M phage antibody library on an excess of the N epitope ibrary (under conditions where infection will not occur). Phages failing to be absorbed will be candidate phage antibodies for the mutated epitope.

     c)   Challenge the M phage antibody library with the N
15   epitope library under stringent lysogenic conditions. Under these conditions the only phage surviving as phage particles will be candidates for those recognising the mutated locus.

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CLAIMS

1. A method for selecting a binding partner for a selector protein from among a library of proteins, which comprises screening the library with a microorganism transformed such that it expresses the selector protein on its surface.

2. A method according to claim 1, wherein the library is expressed on the surface of another transformed microorganism.

3. A method according to claim 2, wherein the respective microorganisms have complementary binding sites, and are transformed by insertion of DNA, respectively encoding the selector protein and the library, into the respective binding sites.

4. A method according to any preceding claim, wherein the selector protein is one of a library expressed by the transformed microorganism.

5. A method according to claim 2, wherein the respective microorganisms are bacterium and phage.

6. A method according to claim 5, wherein the phage lyses the bacterium.

7. A method according to claim 5, whereby the bacterium acquires a resistance phenotype after interaction with the phage.

8. A method according to claim 5, whereby the bacterium expresses a new gene after interaction with the phage.

9. A method according to claim 6, which additionally comprises selecting and amplifying the lytic phage.

10. A method according to claim 7 or claim 8, which additionally comprises selecting and amplifying the lysogenic, resistant or new gene-expressing bacterium.

11. A method according to any of claims 5 to 10, wherein the library or the selector protein is expressed within the phage receptor for bacteria.

12. A method according to any of claims 5 to 11, wherein the library or the selector protein is expressed within the bacterial receptor for phage.

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13. A method according to any of claims 5 to 10,wherein respective libraries are expressed within the phage receptor for bacteria and within the bacterial receptor for phage, and which comprises the additional step of
5   amplifying selected phage/bacterial partners.

14. A method according to any of claims 5 to 10, wherein respective libraries are expressed within the phage receptor for bacteria and within the bacterial receptor for phage, and which comprises the additional step of
10 amplifying non-interacting phage or bacteria.

15. A method according to any of claims 5 to 14, wherein the bacterium and phage are respectively E. coli and bacteriophage λ.

16. A method according to claim 15, wherein the
15 bacteriophage λ expresses antibody within its J gene protein, and the E. coli expresses antigen within its lamB protein.

17. A method according to any of claims 5 to 14, wherein the bacterium includes a F' pilus and the phage is fd
20 (M13,f1) phage.

18. A method according to any of claims 5 to 14, wherein the phage, the bacterium and the respective receptors are selected from T4 gene 37 protein/omp C and T2 gene 38 protein/omp F.

25 19. A method according to any preceding claim, for the selection of agonists or antagonists of growth factors, hormones, lymphokines or any other bioactive molecules, or receptor, or part thereof.

20. A method according to any preceding claim, for the
30 selection of molecules capable of inhibiting the function of (pathogenic) viruses or bacteria.

21. A method according to any preceding claim, for the selection of binding molecules, other than antibodies, useful in diagnostic procedures.

35 22. A method according to any preceding claim, for the selection of inhibitors or activators of the function of the protein, e.g. enzyme.

40

23. A method according to any preceding claim, for the selection of novel enzymes with respect to a reaction intermediate.

24. A method according to any preceding claim, for the selection of interactions between first and second mutated proteins which interacted before mutation but not thereafter.

25. A method according to any preceding claim, for the production of a monoclonal bacterial or phage antibody.

26. A method according to any preceding claim, for the selection of bacterial or phage antibodies against a given coding sequence or library of coding sequences.

27. A method according to any preceding claim, for the selection of phage antibodies or binding proteins recognising members of a gene family specified by one or more conserved motifs.

28. A method according to any preceding claim, for the selection of anti-idiotypic antibodies.

29. A method according to any preceding claim, for the independent selection of first and second binding partners respectively corresponding to chromosomal regions of normal subjects and of those carrying a genetic disease, and which additionally comprises comparing the binding partners.

30. A method according to claim 29, for obtaining (a) phage or other binding molecules to the gene mutations, or clones therefor, or (b) phage antibodies or other binding molecules to the normal counterparts of the mutations, or clones therefor.

31. A method according to any preceding claim, whereby the partner-selector protein binding protects the transformed microorganism from death caused by, for example, phage binding.

32. A method according to any preceding claim, wherein the transformed microorganism is used in a solid phase.

33. A bacterium or phage vector expressing antibody or antigen within a gene thereof.

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34. A bacterium or phage vector according to claim 30,
which is as defined in any of claims 11 to 18.

35. A lambda vector expressing a fusion protein or
antibody within the J gene protein.

5   36. A lambda vector according to claim 33, whose J gene
protein is functional.
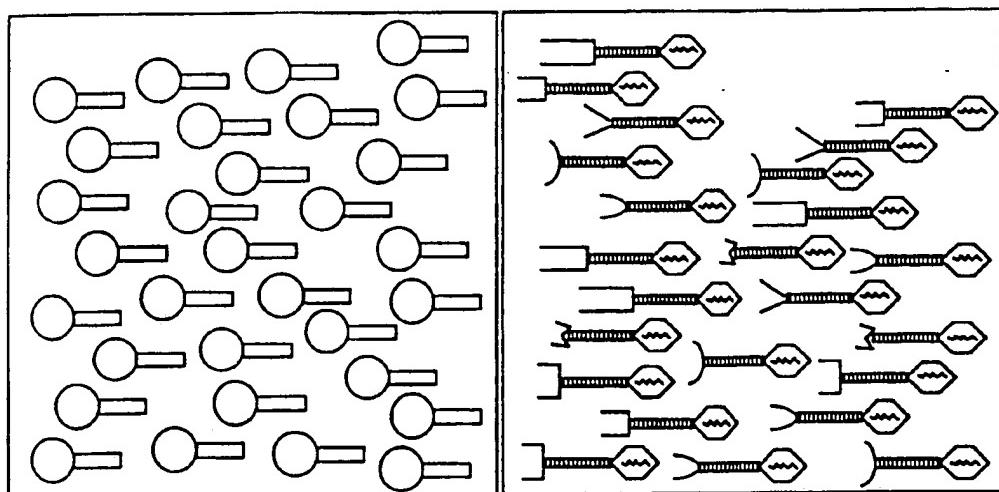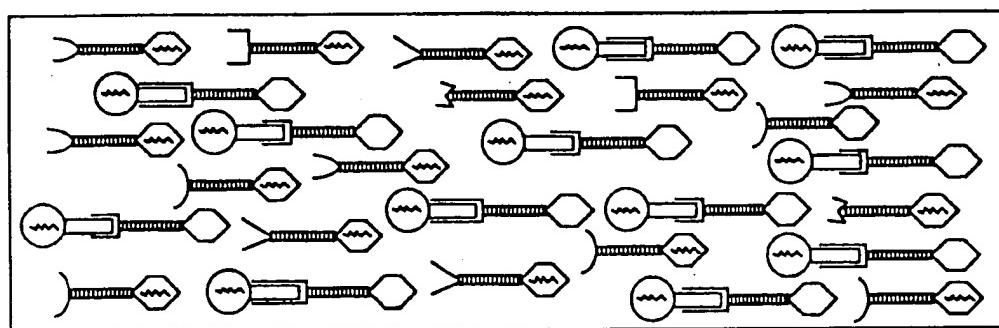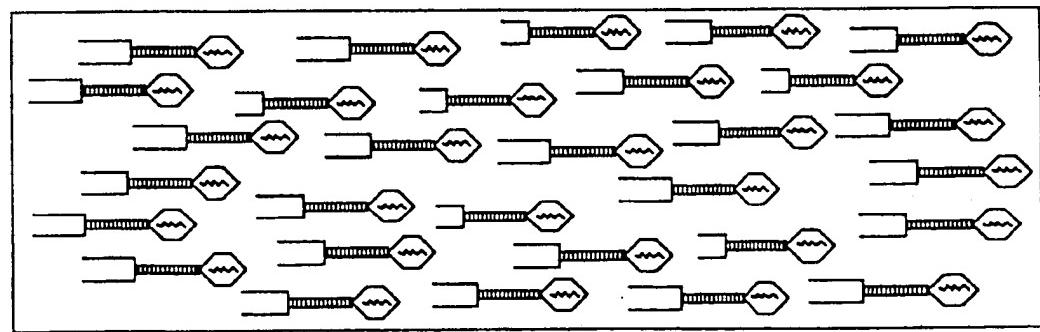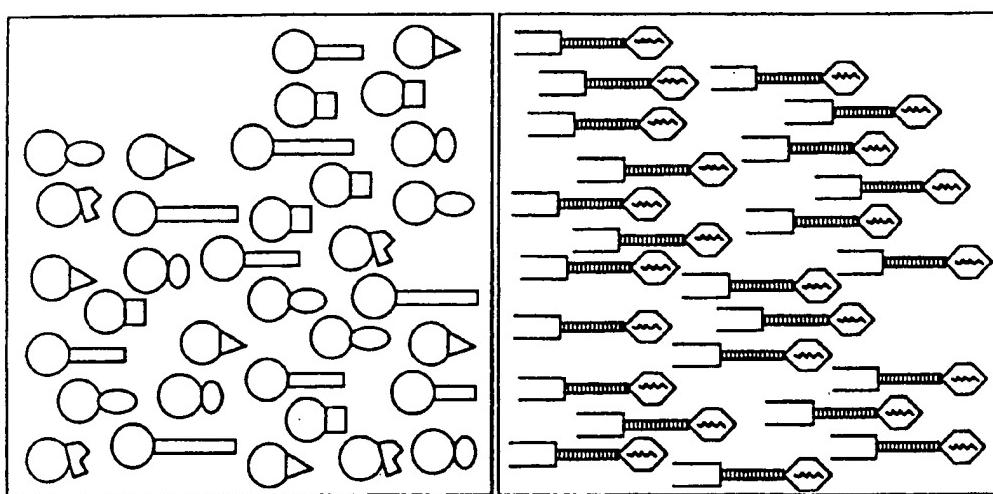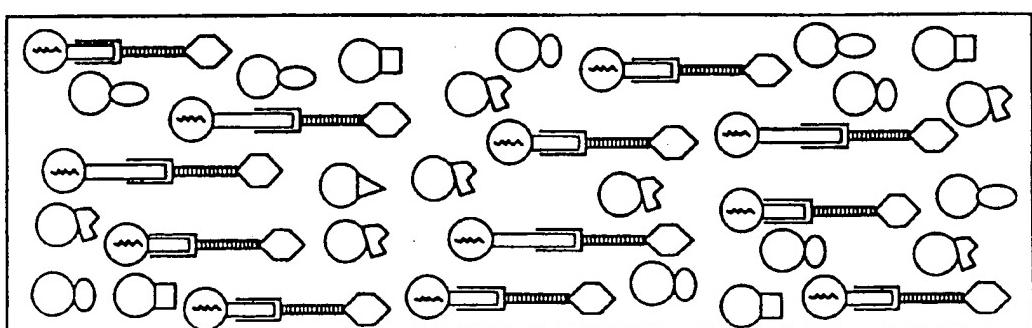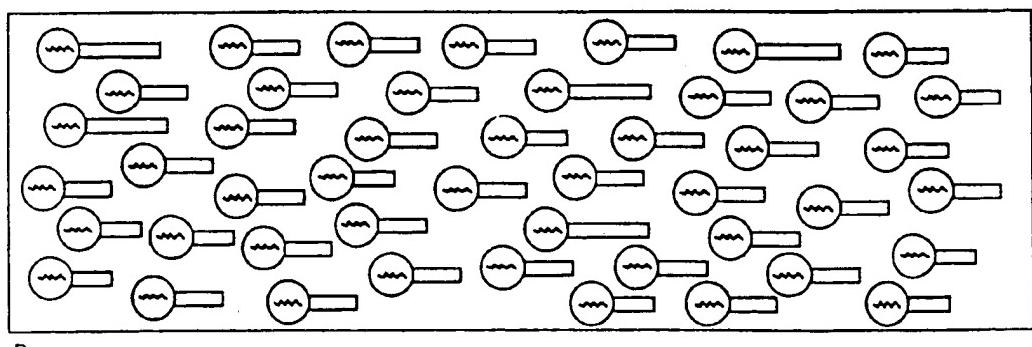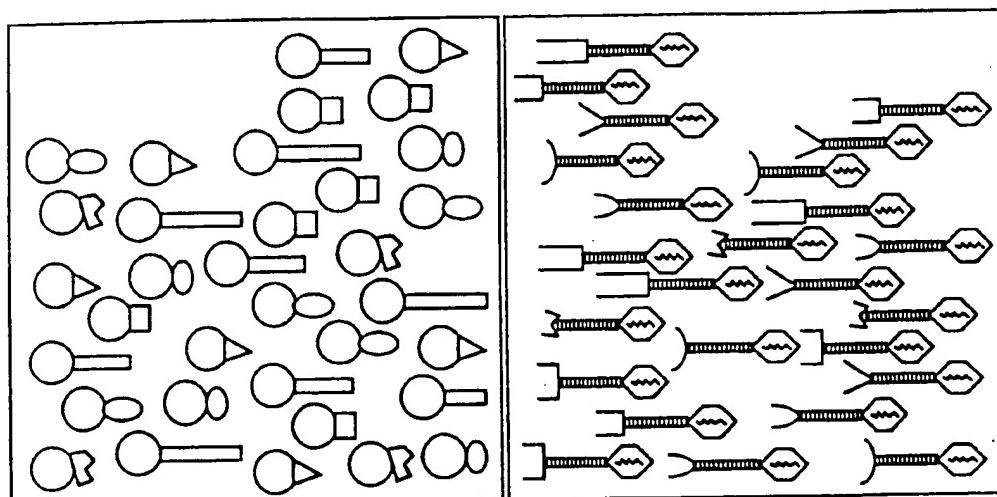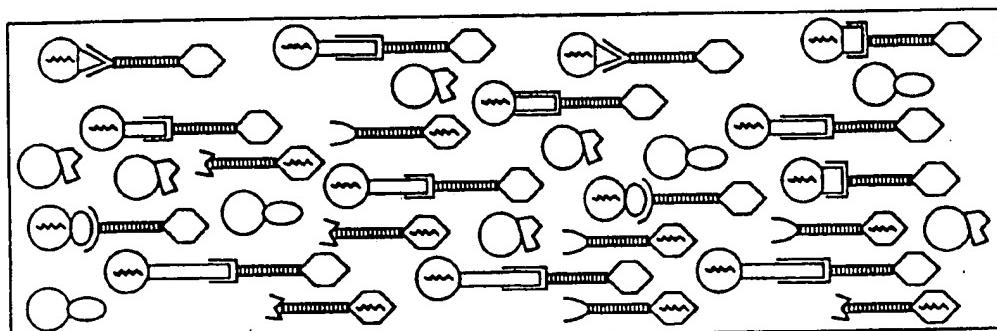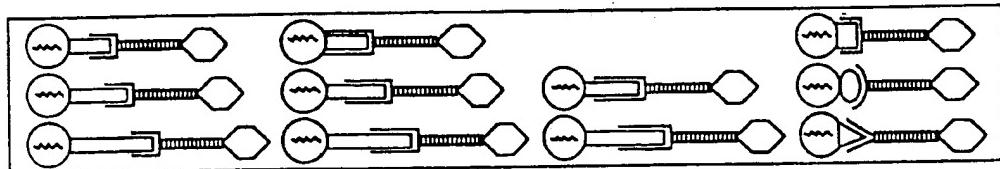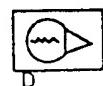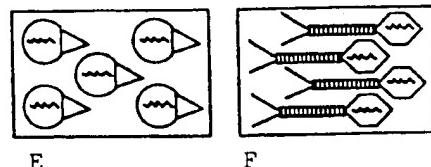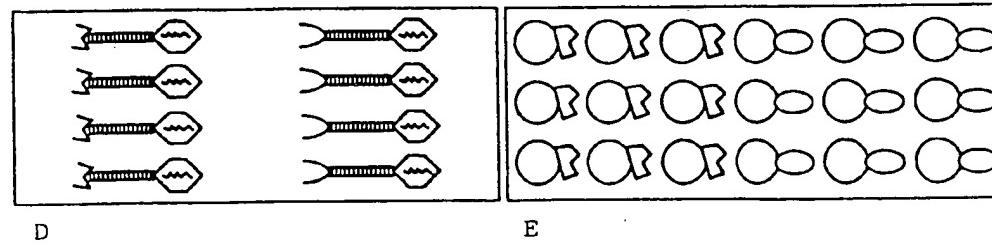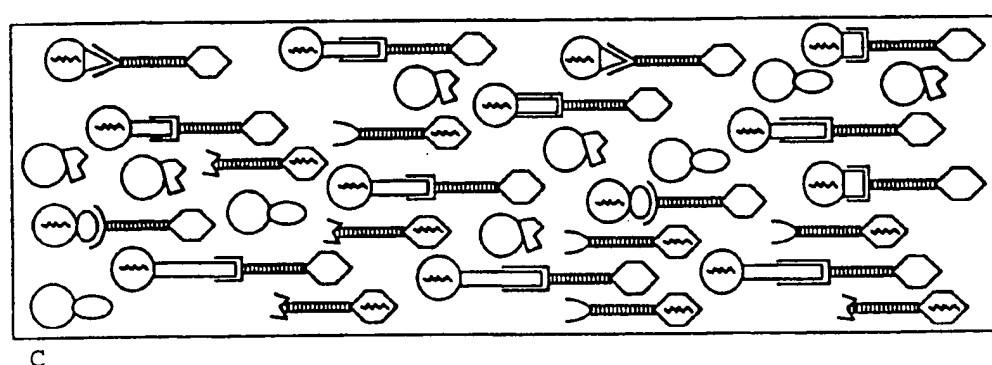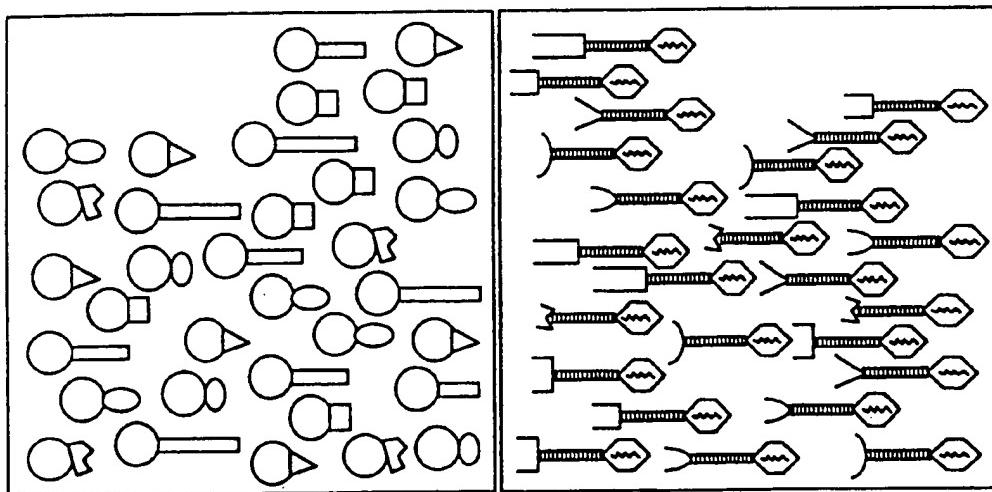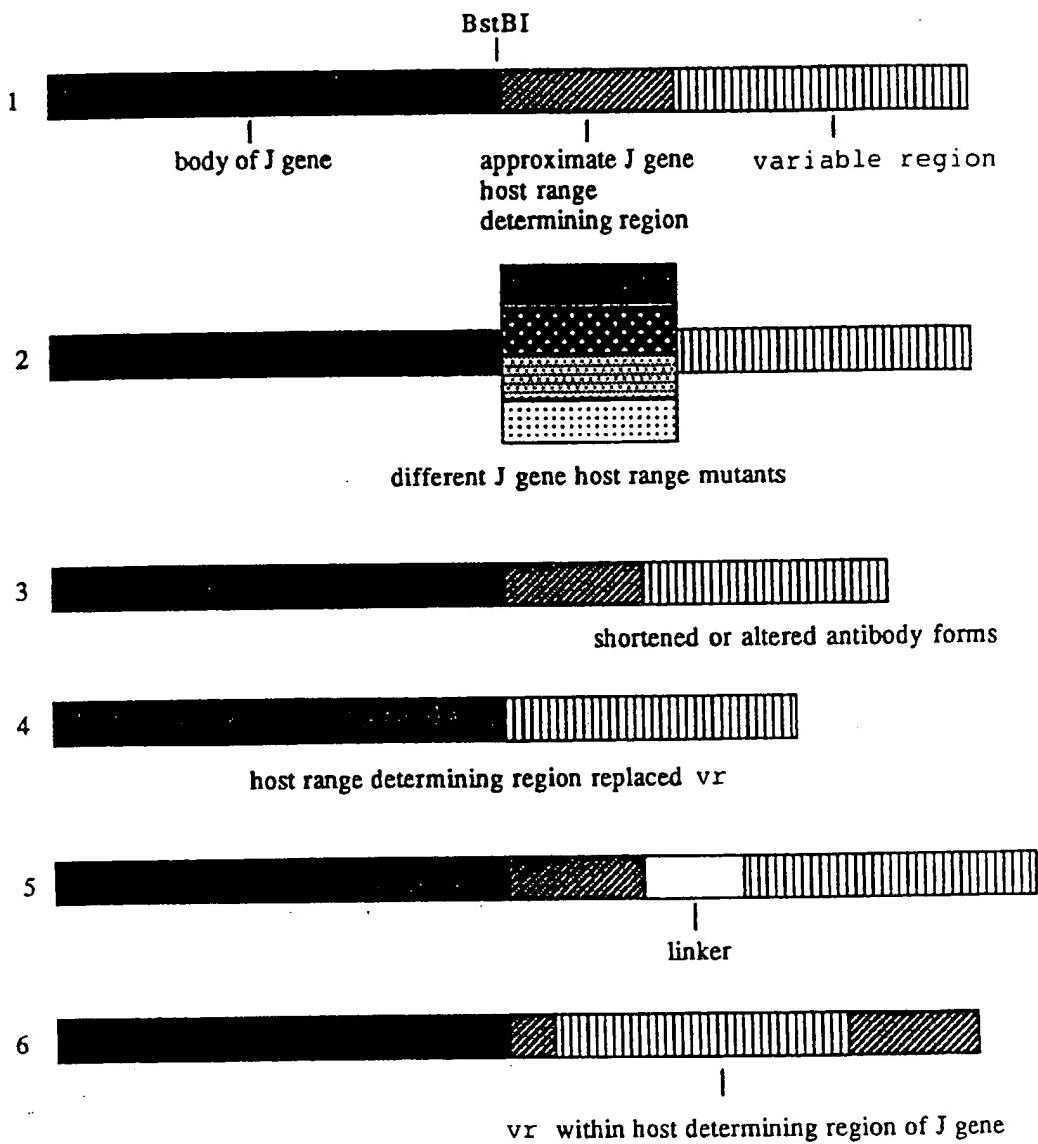
Figure 1

Figure 2

Figure 3

Figure 3b

5/5

BstBI

1

body of J gene | approximate J gene host range determining region | variable region

2

different J gene host range mutants

3

shortened or altered antibody forms

4

host range determining region replaced vr

5

linker

6

vr within host determining region of J gene

figure 4

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.Cl. 5 C12Q1/02; C12N15/73;	G01N33/566; C12N15/13;	C12Q1/70; C12N15/70	G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols		
Int.Cl. 5	C12Q ;	G01N ;	C12N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	SCIENCE. vol. 249, 27 July 1990, LANCASTER, PA US pages 386 - 390; J.K.SCOTT ET AL.: 'Searching for peptide ligands with an epitope library'	33
A	see the whole document especially page 387, left column, lines 1-31; fig. 1; page 389, right column, lines 3-33 ---	1
X	GENE. vol. 70, 30 October 1988, AMSTERDAM NL pages 181 - 189; A.CHARBIT ET AL.: 'Versatility of a vector for expressing foreign polypeptides at the surface of Gram-negative bacteria' cited in the application	34
A	see the whole document ---	1,16
	-/--	

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 APRIL 1992	0 7 MAY 1992
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	
X	GB,A,2 034 717 (NODA INSTITUTE) 11 June 1980 see page 1, line 97 - page 2, line 76 see page 5, line 119 - page 6, line 28; claims ---	33
P,A	WO,A,9 118 980 (CETUS CORPORATION) 12 December 1991 see the whole document ---	1
P,A	WO,A,9 117 271 (AFFYMAX TECHNOLOGIES N.V.) 14 November 1991 see the whole document ---	1
T	NATURE. vol. 352, 15 August 1991, LONDON GB pages 624 - 628; T.CLACKSON ET AL.: 'Making antibody fragment using phage display libraries' ---	

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/04/92

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WO-A-9118980	12-12-91	AU-A-	8081491	31-12-91
WO-A-9117271	14-11-91	AU-A-	7793991	27-11-91